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CDK2 regulation through PI3K and CDK4 is necessary for cell cycle progression of primary rat hepatocytes

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Abstract. *Introduction/Objectives*: Cell cycle progression is driven by the coordinated regulation of cyclin-dependent kinases (CDKs). In response to mitogenic stimuli, CDK4 and CDK2 form complexes with cyclins D and E, respectively, and translocate to the nucleus in the late G_1 phase. It is an on-going discussion whether mammalian cells need both CDK4 and CDK2 kinase activities for induction of S phase. *Methods and results*: In this study, we have explored the role of CDK4 activity during G_1 progression of primary rat hepatocytes. We found that CDK4 activity was restricted by either inhibiting growth factor induced cyclin D1-induction with the PI3K inhibitor LY294002, or by transient transfection with a dominant negative CDK4 mutant. In both cases, we observed reduced CDK2 nuclear translocation and reduced CDK2-Thr160 phosphorylation. Furthermore, reduced pRb hyperphosphorylation and reduced cellular proliferation were observed. Ectopic expression of cyclin D1 alone was not sufficient to induce CDK4 nuclear translocation, CDK2 activity or cell proliferation. *Conclusions*: Thus, epidermal growth factor-induced CDK4 activity was necessary for CDK2 activation and for hepatocyte proliferation. These results also suggest that, in addition to regulating cyclin D1 expression, PI3K is involved in regulation of nuclear shuttling of cyclin-CDK complexes in G_1 phase.

INTRODUCTION

Proliferation of primary hepatocytes has been extensively utilized as a model to study growth factor regulation of cell cycle progression. Because they are non-transformed cells, it is anticipated that this model closely reflects physiological regulatory mechanisms. Hepatocytes undergo transition from G_0 phase to G_1 phase during isolation and plating (Sawada 1989; Labrecque 1994; Loyer *et al*. 1996). In culture, hepatocytes progress through G_1 phase regardless of growth factor stimulation until the restriction point (R-point) in mid-late G_1 phase, beyond which they cannot progress without mitogenic stimulation (Loyer *et al*. 1996). Addition of epidermal

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growth factor (EGF) leads to cyclin D1-induction and complex formation between cyclin D1 and cyclin-dependent kinase 4 (CDK4). The other CDK important for mitogen-induced G_1 progression is CDK2, which complexes with cyclin E. In late G_1 phase, both holoenzymes translocate to the nucleus. CDK protein levels tend to remain constant, but their catalytic activities are tightly regulated. Activation of cyclin D1-CDK4 and cyclin E-CDK2 complexes require phosphorylation of the CDK subunit by the CDK-activating kinase (CAK), on Thr172 and Thr160, respectively (Kato *et al*. 1994). In the nucleus, active cyclin-CDK holoenzymes phosphorylate the retinoblastoma protein (pRb) at specific sites, which results in G_1/S transition (Lundberg & Weinberg 1998).

Two mitogen signalling pathways thought to play important roles in committing quiescent cells into S phase are the Ras-activated Raf/MEK/extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/PDK1/Akt pathways (McCormick & Wittinghofer 1996; McCormick 1999). In fibroblasts, PI3K activity is essential for cyclin D1 accumulation and cyclin D1-CDK4 activity (Diehl *et al*. 1998). Band and coworkers were the first to report a role for PI3K in EGF-induced DNA synthesis of primary rat hepatocytes (Band *et al*. 1999). Later, it was shown that activation of PI3K *via* Grb2-associated binder 2 is necessary and sufficient for proliferation (Kong *et al*. 2000). In line with this, it has been demonstrated that the PI3K cascade is critical for proliferation in hepatocytes, and that inhibition of PI3K activity blocks both cyclin D1 mRNA and protein expression (Coutant *et al*. 2002).

Whether mammalian cells need both CDK2 and CDK4 kinase activity for induction of S phase (Gladden & Diehl 2003; Grim & Clurman 2003; Kozar *et al*. 2004; Malumbres *et al*. 2004; Sherr & Roberts 2004; Su & Stumpff 2004; Sanchez & Dynlacht 2005) has been in dispute. Other CDKs may compensate for the ablation of a particular cyclin-CDK complex in the context of knock-out mice. Whether this can also occur if CDKs are knocked out after cellular differentiation remains unexplored. In cultured cells, it has been suggested that phosphorylation of pRb by cyclin D-CDK4 initiates a subsequent round of phosphorylation of the tumour suppressor that is completed by cyclin E-CDK2 (Lundberg & Weinberg 1998). This suggests that CDK4 and CDK2 do not have overlapping functions in cell culture.

The aim of this work has been to clarify molecular mechanisms involved in CDK regulation of G_1/S transition in primary rat hepatocytes. In particular, we wanted to explore signalling pathways regulating CDK2 activity, information concerning that has been lacking. We found that cyclin E was induced independently of growth factor stimulation and prior to cyclin D1. CDK2 function depended on EGF-induced CDK4 activity, as well as that of PI3K. Ectopic expression of cyclin D1 was not sufficient alone for nuclear translocation of CDK4 or CDK2, pRb hyperphosphorylation or for cell proliferation.

MATERIALS AND METHODS

Materials

Williams medium E, Dulbecco's modified Eagle's medium, penicillin and streptomycin were from Gibco (Grand Island, NY, USA). Collagenase (C-0130), collagen (C-7661), dexamethasone (D-4902), EGF (E-1257) and insulin (I-6634) were obtained from Sigma Aldrich (St. Louis, MO, USA). Plastic culture dishes were purchased from Sarstedt (Nümbrecht, Germany). LY294002 was from Calbiochem (La Jolla, CA, USA). Thymidine was obtained from GE Healthcare (London, UK). Dynabeads Protein G was purchased from Dynal Biotech (Oslo, Norway).

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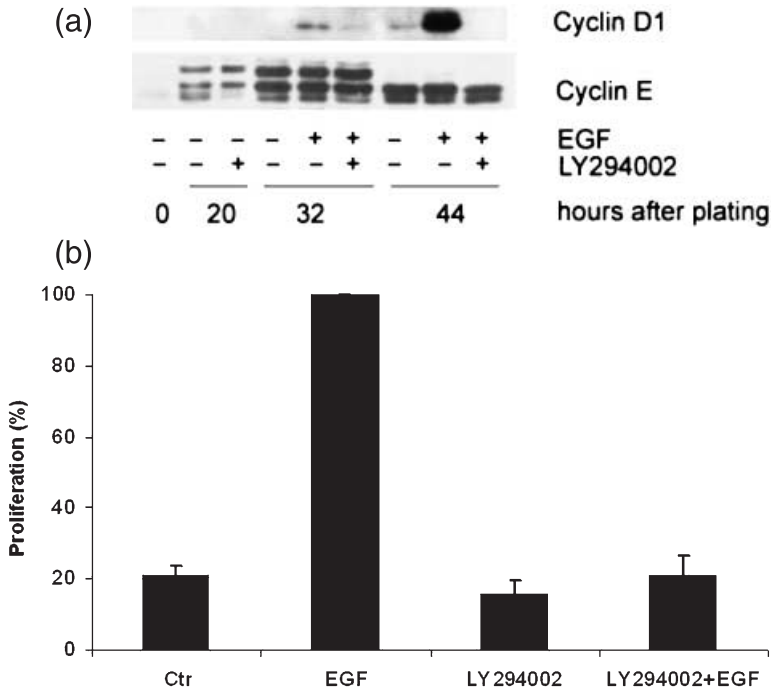

Figure 1. Effects of PI3K inhibition. Primary cultures of hepatocytes were incubated with or without LY294002 prior to epidermal growth factor (EGF) stimulation and were harvested at different time points thereafter. (a) Western immunoblot analysis was performed on lysates with antisera to cyclin D1 and cyclin E. (b) Hepatocytes were harvested 30 h after EGF stimulation. Thymidine incorporation was measured by scintillation counting. The results represent an average of three separate experiments.

Cell isolation and culture

Young adult male Wistar rats (Møllergård and Bomhoff, Odense, Denmark), weighing 200–220 g, were kept on a 12-h light:dark cycle and were fed *ad libitum*. Hepatocytes were isolated in a two-step *in vitro* version (Seglen 1976) of the collagenase perfusion technique (Berry & Friend 1969) with modification (Christoffersen *et al*. 1984). Cell death was measured using the trypan blue exclusion technique. Cells were seeded at density of 20 000 cells/cm^2. Serum-free culture medium consisted of a 1 : 1 combination of Williams Medium E and Dulbecco's modified Eagle's medium, with a final glucose concentration of 8.4 mM. Medium was supplemented with penicillin (67 μg/mL), streptomycin (100 μg/mL), collagen (3 μg/mL), dexamethasone (25 nM) and insulin (100 nM). Cultures were maintained in a 5% CO_2 atmosphere at 37 °C. Hepatocytes were exposed to 10 nM EGF. For inhibition assays, cells were pre-incubated with 10 μM LY294002 1 h prior to EGF-stimulation, except in induction studies of cyclin D1 and cyclin E (Fig. 1a), where LY294002 was added at the time of plating.

Western immunoblot analysis

Cultured hepatocytes were lysed in Tris-lysis buffer, pH 7.4, with 60 mM Tris-HCl, 10% glycerol, 3% sodium dodecyl sulfate (SDS), 1 M EDTA, 0.2 mM AEBSF, 20 μM leupeptin, 200 units/mL aprotinin, 65 μM sodium orthovanadate and 10 mM β-glycerophosphate. Lysates were sonicated. Protein concentrations of all samples were measured by DC protein assay (Bio-Rad, Hercules,

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CA, USA), and were adjusted to equal concentrations in the lysis buffer containing 5% β-mercaptoethanol and 0.0025% bromophenol blue. Protein samples were boiled for 4 min and were stored at –20 °C. Proteins were separated by SDS-PAGE and were electrotransferred to nitrocellulose membranes (GE Healthcare) for subsequent protein detection as described (Laemmli 1970; Towbin *et al*. 1979). Filters were blocked in Tris-buffered saline containing 5% fat-free dry milk and were incubated with primary antibodies diluted in 1% fat-free dry milk in Tris-buffered saline over night at 4 °C. The following primary antibodies were used: rabbit anti-Ser807/811 phosphorylated Rb and anti-Thr160 phosphorylated CDK2 from Cell Signalling Technology (Beverly, MA, USA), rabbit anticyclin D1 from Upstate Biotechnology (Lake Placid, NY, USA), rabbit anti-CDK4 from Santa Cruz Biotechnology (Santa Cruz, CA, USA), mouse anti-HA from Covance (Berkely, CA, USA), rabbit anti-Thr826 phosphorylated Rb and anti-Cyclin E from Abcam (Cambridge, UK) and mouse anti-β-tubulin from Sigma Aldrich (St. Louis, MO, USA). After washing, filters were incubated with horse radish peroxidase (HRP)-conjugated goat antirabbit IgG and donkey antimouse IgG from Sigma Aldrich at room-temperature for 90 min. Immunobinding was detected by the enhanced chemiluminescence (ECL) method plus exposure to Hyperfilm-ECL (GE Healthcare).

Measurement of DNA synthesis

For thymidine incorporation, cells were cultured in 6-well plates and were grown as described above. [^{3}H]-thymidine (1 μCi/mL) was added to the cultures 48 h after plating. DNA synthesis was assessed by determining the amount of radioactivity incorporated into DNA. The cellular material was precipitated with 2 mL 5% trichloroacetic acid for 2×10 min. Acid-precipitated material was dissolved in 0.6 mL 1 M KOH, followed by liquid scintillation counting. Protein was measured as described earlier.

Rat hepatocyte transfection

Transfection was carried out with DOTAP reagent (Biontex Laboratories, Martinsried, Munich, Germany) according to the manufacturer's instructions. The HA-tagged CDK4^{N158} construct was a kind gift from Juan Zalvides laboratory and the wild-type cyclin D1 plasmid was kindly provided by J. Alan Diehl. The pEYFP-Mem vector (Clontech, Mountain View, CA, USA) is one such that encodes a fusion protein consisting of the N-terminal 20 amino acids of neuromodulin and a yellow–green fluorescent variant of the enhanced green fluorescent protein. EGF was added 15 h after transfections and cells were harvested at different time points following growth factor addition. Fluorescence microscopy counting of hepatocyte cultures transfected with pEYFP-Mem indicated a transfection efficiency of around 40%.

Immunocytochemistry and confocal immunofluorescence microscopy

Cells were washed in phosphate-buffered saline, rinsed in dH_2O and were fixed in ethanol for 10 min. Washed and air-dried cells were incubated overnight with the following antibodies: rabbit anti-CDK4 and anti-CDK2 from Santa Cruz Biotechnology, mouse anticyclinD1 from NeoMarkers (Fremont, CA, USA) and rabbit anticyclin E from Sigma Aldrich, diluted in phosphate-buffered saline containing 1% bovine serum albumin. Immunobinding was detected with Cy3-conjugated donkey antirabbit IgG or RRX-conjugated donkey antimouse IgG antibodies from Jackson Immunoresearch (West Grove, PA, USA). Cells were mounted in Dako mounting medium (Dako, Carpinteria, CA, USA). As controls for cross-reactivity of the secondary antibody, primary antibody was omitted from the staining sequence. Stained cells were examined with a Leica TCS SP confocal microscope (Leica, Heidelberg, Germany) equipped with Ar (488 nm) and two He/Ne (543 and 633 nm) lasers and LCS software.

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RESULTS

Cyclin E was induced independently of EGF stimulation and PI3K activation

Cyclin D1 is up-regulated during the G_1 phase in proliferating hepatocytes in culture and its induction is dependent on PI3K signalling (Lu *et al*. 1992; Albrecht *et al*. 1993; Albrecht *et al*. 1995; Loyer *et al*. 1996; Rickheim *et al*. 2002). Mechanisms regulating cyclin E induction are not as well characterized; thus, we compared cyclin D1 and cyclin E protein expression in unstimulated and in EGF-stimulated hepatocytes, exposed to the PI3K inhibitor LY294002 or not (Fig. 1a). Western immunoblotting demonstrated that cyclin E was induced about 20 h after cell plating, independent of growth factor stimulation. Initially, three cyclin E bands (isoforms of 50–55 kDa) were observed. Expression of the largest isoform disappeared about 44 h after plating in both unstimulated and EGF-stimulated cells. Exposure to PI3K inhibitor did not affect cyclin E expression. Conversely, cyclin D1 was only induced in EGF-stimulated cultures, and appeared about 12 h after EGF-stimulation. In agreement with previous reports (Coutant *et al*. 2002; Rickheim *et al*. 2002), we found that inhibition of PI3K abrogated cyclin D1 induction. In addition, in accordance with published observations, we found that PI3K-inhibition almost completely abolished hepatocyte proliferation (Fig. 1b).

PI3K activity promoted nuclear translocation and Thr160 phosphorylation of CDK2

Because cyclin E induction was independent of growth factor stimulation, we wanted to study how EGF affected its catalytic partner CDK2. Cellular trafficking of G_1 cyclins and CDKs was investigated by immunocytochemistry. CDK2 and Cyclin E were present in both unstimulated and EGF-stimulated hepatocytes, whereas nuclear translocation was only observed in the latter (Fig. 2a). Inhibition of PI3K did not affect cyclin E induction, but abrogated both CDK2 and cyclin E nuclear translocation. We also demonstrated CDK4 to be located in the cytoplasm in unstimulated cells, but following EGF-stimulation, both CDK4 and cyclin D1 accumulated in the nucleus. When cyclin D1-induction was inhibited by LY294002, CDK4 remained in the cytoplasm.

CDK2 activity is regulated by CAK-phosphorylation of its Thr160 site (Poon *et al*. 1994). Because CAK is located in the cell nucleus (Tassan *et al*. 1994; Jordan *et al*. 1997), we did not anticipate CDK2 phosphorylation in PI3K inhibited cells. Western immunoblotting analysis of CDK2-Thr160 phosphorylation revealed that CDK2 remained inactivated (Fig. 2b). EGF-induced pRb phosphorylation at CDK2-preferential sites (Ser807/811) (Brugarolas *et al*. 1999) was also clearly reduced in PI3K inhibited cells. Thus, cyclin D1 induction, nuclear accumulation of CDK4, CDK2 and cyclin E and CDK2 phosphorylation, were all dependent on PI3K signalling.

CDK4 activity was necessary for nuclear translocation of CDK2

As PI3K-inhibited cells showed cytoplasmic retention of both CDK4 and CDK2 even in the presence of EGF, we further examined whether CDK2 functions depended on cyclin D1 induction and CDK4 activity. Cells were transfected with a dominant negative, N158-mutated CDK4 ($CDK4^{N158}$) or a control vector, pEYFP-Mem. Immunocytochemical staining revealed that EGF-induced nuclear translocation of CDK2 was reduced in $CDK4^{N158}$ transfected cells compared to cells transfected with pEYFP-Mem (Fig. 3a, upper panel). Furthermore, cyclin E showed reduced nuclear import in these cells (Fig. 3a, lower panel). Cyclin D1 and CDK4 nuclear accumulation were not affected by $CDK4^{N158}$-transfection (data not shown).

The biological effects of CDK4 are mediated through phosphorylation of target proteins, such as pRb (Kato *et al*. 1993). Phosphorylation of pRb was therefore examined in EGF-stimulated

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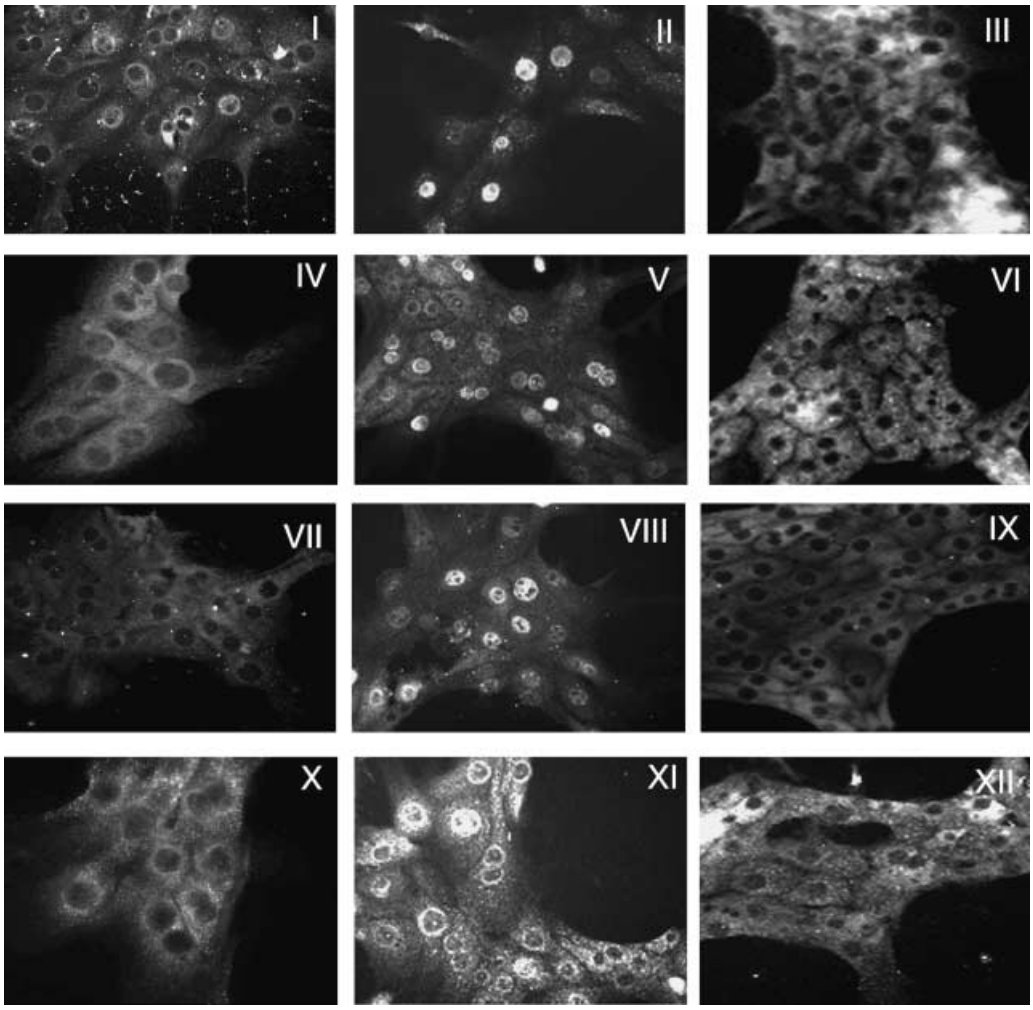

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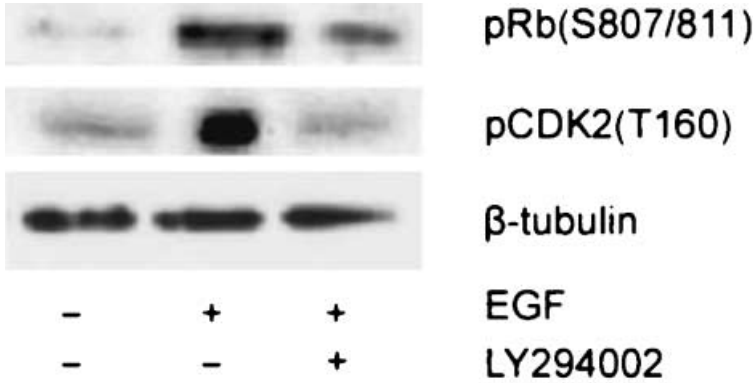

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cells overexpressing $CDK4^{N158}$. Western immunoblotting demonstrated reduced pRb phosphorylation at both the CDK4-preferential site (Thr826) (Zarkowska & Mittnacht 1997) and the CDK2-preferential sites in $CDK4^{N158}$-transfected cells (Fig. 3b). Furthermore, overexpression of $CDK4^{N158}$ also reduced CDK2-Thr160 phosphorylation. Thymidine incorporation experiments demonstrated reduced proliferation in $CDK4^{N158}$ expressing cells compared to that of transfected control cells (Fig. 3c), implying that EGF-induced CDK4 activity was necessary for subsequent CDK2 activation and hepatocyte proliferation.

Ectopic cyclin D1 expression did not induce CDK2 activation or proliferation

Because our results indicated that PI3K activity was necessary for both cyclin D1-induction and CDK2 regulation, we wanted to explore whether cyclin D1 expression alone was sufficient for nuclear translocation of G_1 CDKs and cell proliferation. Following tritiated thymidine incorporation, cell proliferation was not induced when cyclin D1 was ectopically expressed (Fig. 4a), suggesting that additional signalling was necessary for cell cycle progression. Upon EGF-stimulation, increased proliferation was seen in both control transfected and cyclin D1 wild-type transfected hepatocytes. Cells overexpressing cyclin D1 and stimulated with EGF, demonstrated strong growth inhibition when exposed to the PI3K inhibitor LY294002.

Also, intracellular localization of wild-type cyclin D1 was examined by immunofluorescence microscopy. Nuclear accumulation of ectopically expressed cyclin D1 was not observed in unstimulated cells. Upon EGF stimulation, nuclear staining of wild-type cyclin D1 was observed. This nuclear accumulation was abolished in response to PI3K inhibition (Fig. 4b, upper panel). Nuclear translocation of CDK4 or CDK2 was not observed in unstimulated cells overexpressing cyclin D1, but both kinases were accumulated in the nucleus following EGF-stimulation. EGF-induced nuclear translocation of CDK2 and CDK4 was not observed when PI3K was inhibited (Fig. 4b, middle and lower panels). Thus, it appeared that PI3K regulated CDK-activity by at least two mechanisms: one by the induction of cyclin D1 and the other by regulating nuclear import of cyclin-CDK complexes.

To further confirm the role of PI3K during nuclear translocation of CDK2, activation of CDK2 was studied by Western immunoblot analysis. CDK2 remained unphosphorylated at Thr160 in unstimulated cells overexpressing cyclin D1, but was phosphorylated after EGF-stimulation. EGF-induced phosphorylation of CDK2 was inhibited when cells were exposed to LY294002. Similarly, pRb remained hypophosphorylated in these situations (Fig. 4c).

Taken together, our results have demonstrated that CDK4 activity was necessary for nuclear translocation and activation of CDK2 and for hepatocyte proliferation, in a PI3K dependent process. Induction of cyclin D1 alone was not sufficient for activation of CDK4 and CDK2, indicating that PI3K has a dual role in cell cycle progression. We suggest that, in addition to regulation of cyclin D1-induction, PI3K is involved in regulation of nuclear shuttling of cyclin-CDK complexes in G_1 phase.

Figure 2. Reduced nuclear accumulation of CDK2, CDK4, cyclin D1 and cyclin E after PI3K inhibition. (a) Immunofluorescence staining of unstimulated hepatocytes (I, IV, VII, X) and hepatocytes 30 h after epidermal growth factor (EGF) stimulation, pre-incubated with LY294002 (III, VI, IX, XII) or not (II, V, VIII, XI). Antibodies recognizing CDK2 (I–III), CDK4 (IV–VI), cyclin D1 (VII–IX) and cyclin E (X–XII) were used (40× objective, zoom 1×). (b) Western immunoblotting was performed on lysates from control cells, from EGF-stimulated cells and from hepatocytes exposed to LY294002 and EGF for 30 h. Antibodies to pRb(S807/811) and pCDK2(T160) were used. Protein expression of β-tubulin was used as a loading control. Representative examples of at least three experiments are shown.

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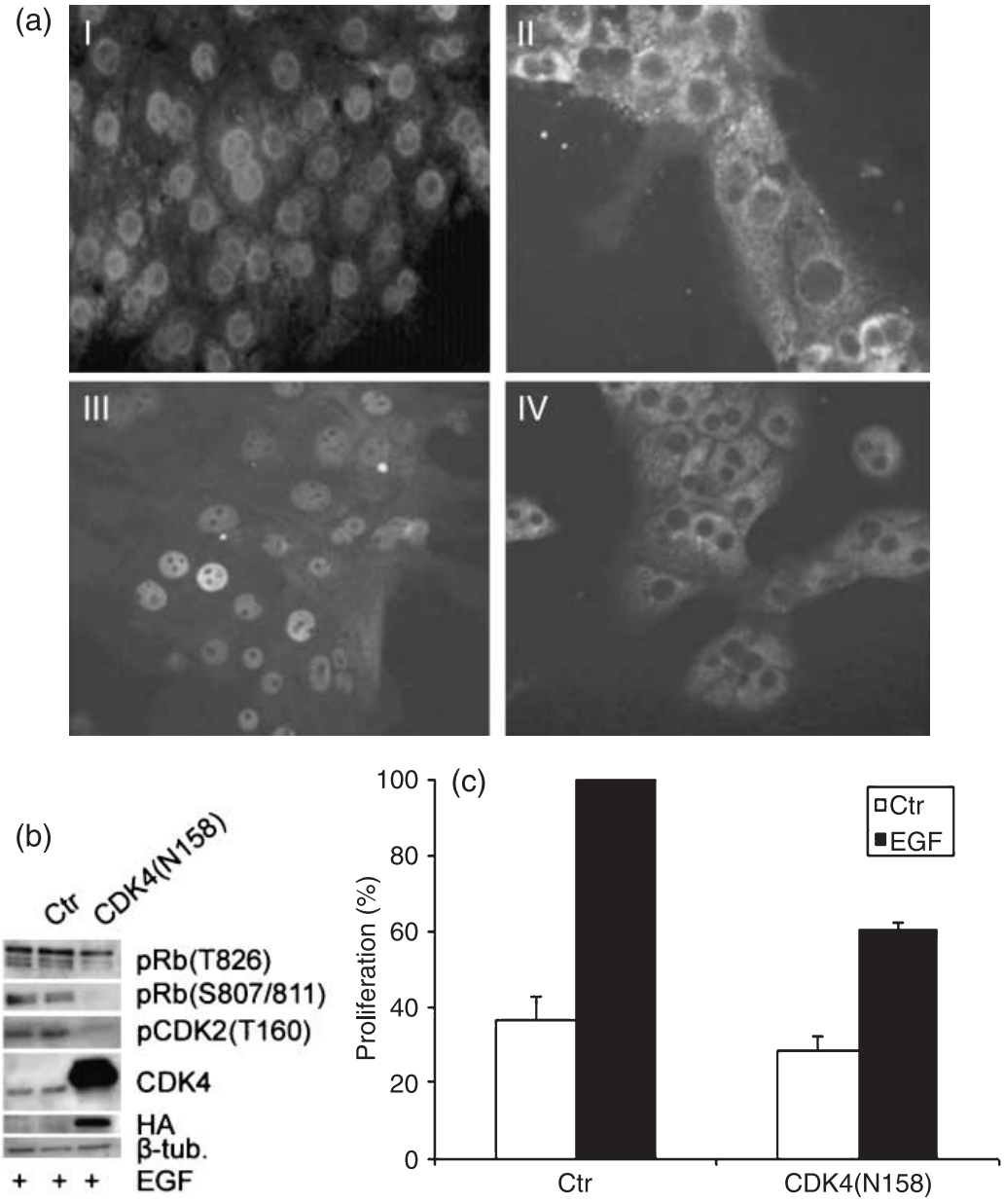

Figure 3. Effects of reduced CDK4 activity. Primary cultures of hepatocytes were either transfected with a dominant negative mutant of CDK4 ($CDK4^{N158}$) or with a control vector. After 15 h, cells were stimulated with epidermal growth factor (EGF). (a) Immunofluorescence staining of hepatocytes, 30 h after EGF stimulation. Control transfected cells (I, III) and $CDK4^{N158}$ expressing cells (II, IV) were stained with antibodies that recognize CDK2 (I–II) and cyclin E (III–IV). Representative images of at least three experiments are shown (40× objective, zoom 1×). (b) Hepatocytes were harvested 30 h after EGF stimulation. Western immunoblotting analysis was performed on lysates with antibodies that recognized Rb phosphorylated either on Thr826 or Ser807/811. Antisera to CDK4 and HA were used to detect expression of transfected $CDK4^{N158}$ and protein expression of β-tubulin was used as a loading control. (c) Cells were harvested 30 h after EGF addition, and accumulated tritiated thymidine incorporation was measured by scintillation counting. The results represent a combination of three separate experiments.

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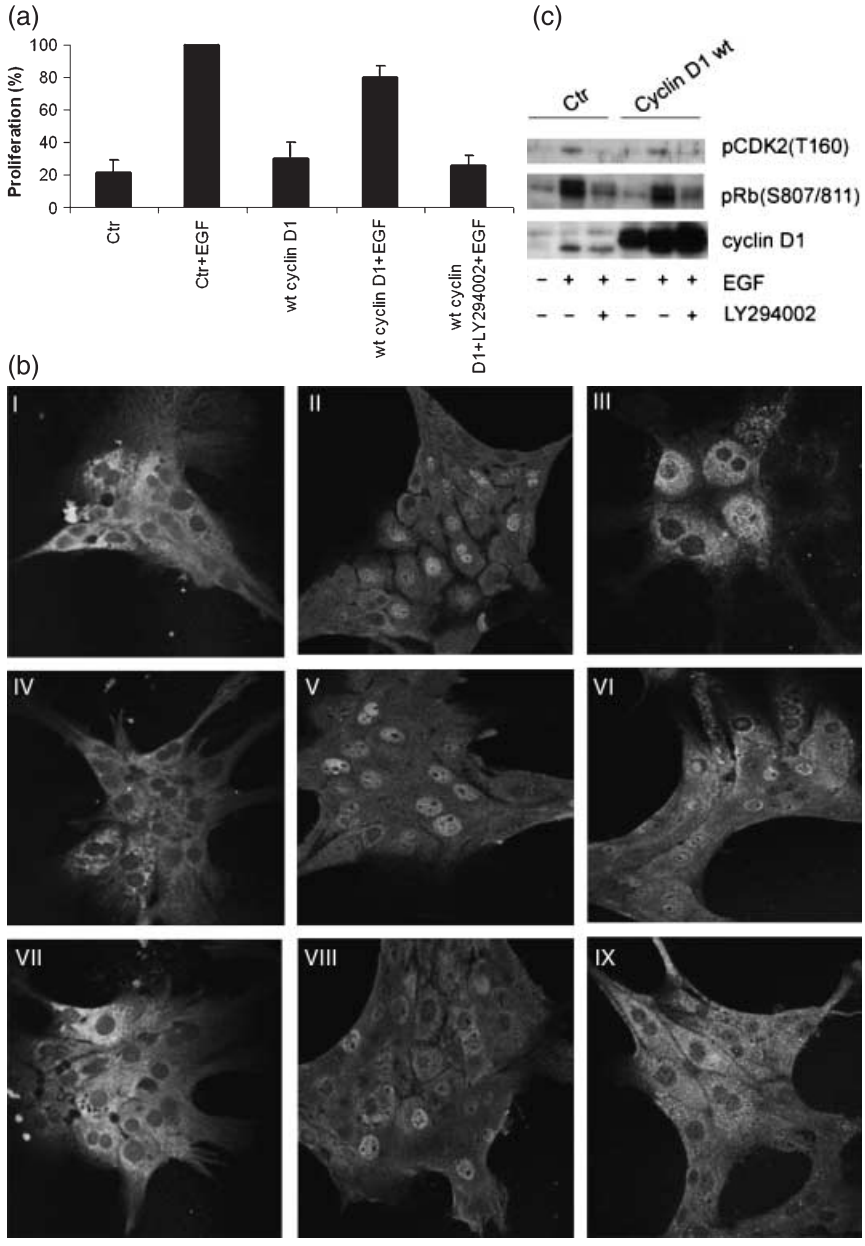

Figure 4. Ectopic expression of cyclin D1 did not induce proliferation. Primary cultures of hepatocytes were transfected with wild-type cyclin D1 or with a control vector. After 15 h, cells were incubated with or without LY294002 prior to epidermal growth factor (EGF) stimulation. Cells were harvested 30 h after EGF addition. (a) Accumulated tritiated thymidine incorporation was measured by scintillation counting. The results represent a combination of three separate experiments. (b) Immunofluorescence staining of hepatocytes transfected with wild-type cyclin D1 (I, IV, VII), transfected with wild-type cyclin D1 and exposed to EGF (II, V, VIII), and hepatocytes transfected with wild-type cyclin D1 and exposed to LY294002 prior to EGF (III, VI, IX). Antibodies recognizing CDK2 (I–III), CDK4 (IV–VI) and cyclin D1 (VII–IX) were used (40× objective, zoom 1×). (c) Hepatocytes were harvested and subjected to Western immunoblotting with antisera to pCDK2(T160) and pRb(S807/811). Antiserum to cyclin D1 was used as a positive control of transfection. Representative examples of at least three experiments are shown.

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DISCUSSION

In adult liver, hepatocytes largely are resting cells and growth induction involves an initial priming step. Then, the cells acquire competence for growth factor-induced cell cycle progression; primary hepatocyte proliferation closely resembles this pattern. This appears as hepatocyte isolation and plating induce the priming phase. Further cell cycle progression requires mitogen stimulation, and EGF, transforming growth factor α and hepatocyte growth factor are strong inducers of hepatocyte proliferation (Fausto 2000).

In studies of mammalian cells, the D-type cyclins seem to act as intracellular 'sensors' of extracellular stimuli that promote proliferation during the G_1 phase (Sherr & Roberts 1999; Sherr 2000). In hepatocytes, cyclin D1 is a pivotal mediator of G_1 progression in response to EGF, insulin or PI3K signalling, whereas cyclin D2 and cyclin D3 appear not to play critical roles there (Albrecht *et al*. 1999; Rickheim *et al*. 2002). We have confirmed that induction of cyclin D1 was EGF-dependent, and inhibition of the PI3K pathway abolished cyclin D1 expression, as well as hepatocyte proliferation. Growth factor regulation of cyclin E and of CDK2 function is not as well characterized; thus, the aim of the current study was to characterize growth factor effects on CDK2 activation and to determine whether these effects were mediated through CDK4. We found cyclin E protein to be induced in both unstimulated and EGF-stimulated hepatocytes prior to cyclin D1 expression. Inhibition of the PI3K pathway did not reduce cyclin E induction. Thus, the mechanism responsible for growth factor-independent cyclin E induction in G_1 phase appeared to be a constituent of the priming phase. In the absence of growth factor stimulation, CDK2 and cyclin E resided in the cytoplasm. Furthermore, EGF-stimulated nuclear translocation of CDK2 and cyclin E was attenuated by PI3K inhibition. CDK2 and CDK4 are activated by CAK, which is constitutively active and resides in the nucleus (Matsuoka *et al*. 1994; Tassan *et al*. 1994). Thus, cytoplasmic retention of CDK2 and cyclin E may explain why CDK2 remained unphosphorylated at Thr160, and pRb remained hypophosphorylated in PI3K-inhibited cells.

Because PI3K inhibition attenuated both cyclin D1 induction and CDK2 activity, we explored whether the latter effect was mediated through CDK4. Ectopic expression of a dominant negative, kinase defective CDK4 led to inhibited nuclear translocation of CDK2 and cyclin E, abridged Thr160 phosphorylation of CDK2 and reduced proliferation. This suggested that CDK2 activity relied on prior CDK4 activation. However, it has previously been shown that overexpression of $CDK4^{N158}$ did not induce growth arrest in U2OS-cells (van den Heuvel & Harlow 1993). Similarly, it has been demonstrated that cyclin E-CDK2 can phosphorylate pRb in the absence of CDK4-cyclin D1 activity in IIC9-cells, suggesting that phosphorylation of pRb by cyclin E-CDK2 is not dependent on prior cyclin D1-CDK4 activity (Keenan *et al*. 2004); use of primary cells, rather than cell-lines, can possibly explain the discrepancy between our model and others. Alternatively, CDK4 activity is required in cells that are recruited into the cell cycle from a resting (G_0) state.

Nelsen and coworkers have demonstrated that transient expression of cyclin D1 is sufficient to promote hepatocyte replication and liver growth (Nelsen *et al*. 2001). Conversely, we found that ectopic cyclin D1 expression did not induce CDK4 nuclear translocation, CDK2 activation or cell proliferation in the absence of growth factor. EGF-stimulation through PI3K was required even in this situation. Thus, it appeared that in addition to cyclin D1 induction, PI3K activity was necessary for translocation of cyclin D1-CDK4 complexes to the nucleus, and that this process was a prerequisite for nuclear translocation and functional activation of CDK2. Whether these differences reflects differences between our cell culture model and liver regeneration in an

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intact organ, or can be attributed to the adenoviral transfection method applied in the latter, remains unresolved.

Our data support the notion that nuclear trafficking of CDK2 and CDK4 is strictly regulated and under mitogen control. Whereas cyclin E has its own nuclear localization sequence, cyclin D1 has not. Thus, nuclear translocation of cyclin D1-CDK4 is thought to rely on other proteins. Neither CDK contains a nuclear localization sequence, and depends on the interaction with cyclin E to reach the nucleus (Diehl & Sherr 1997). However, lately it has been demonstrated that nuclear localization signal in the N-terminus of cyclin E is apparently non-functional (Kelly *et al*. 1998; Geisen & Moroy 2002). These results, in addition to our observations, indicate that nuclear translocation of both cyclin D1-CDK4 and cyclin E-CDK2 complexes is mediated by binding of a partner protein, maybe in a cell cycle-dependent manner.

In summary, our results suggested that cyclin E induction in primary rat hepatocytes was associated with recruitment into the cell cycle during the priming phase. In G_1 phase, growth factor regulated cell cycle progression through cyclin D1 induction and subsequent CDK4 activation, and through PI3K, regulated trafficking of CDK2 and CDK4. CDK4 activity was necessary for CDK2 nuclear translocation, activation and cell proliferation, in a process initiated by PI3K. Induction of cyclin D1 was not sufficient alone to induce CDK4 and a subsequently CDK2 activity, indicating that PI3K has a dual role in hepatocyte cell cycle progression.

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